A Clearing Protocol for Whole Tissues: An Example Using Haustoria of Orobanchaceae

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Protocol Note

A clearing protocol for whole tissues: An example using haustoria of Orobanchaceae[1]

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- *Premise of the study:* Due to lack of success in clearing whole tissues using only classical clearing techniques (e.g., Herr's 4½ solution, KOH, NaOH, lactic acid saturated with chloral hydrate), and because tissue degradation is often a result of harsh clearing agents (e.g., KOH, NaOH), a novel combined treatment was sought to improve the removal of obscuring tannins from intact haustoria.
- *Methods and Results:* Stockwell's bleach proved to be useful in removing tannins from haustoria, usually within 3 d (up to 10 d), rendering them opaque to (rarely) translucent. After bleaching, haustoria were successfully cleared in 1–3 d in a solution of lactic acid saturated with chloral hydrate at 42°C.
- *Conclusions:* The two-step clearing protocol reported here will now facilitate structural studies on haustoria, such as those examining the presence and distribution of callose, and three-dimensional reconstruction using confocal microscopy. Tissues in this study did not suffer from the degradation in quality observed using harsher treatments. This protocol should be useful for other difficult-to-clear tissues that are unable to be cleared using classical protocols alone.

Key words: chloral hydrate; clearing; haustorium; lactic acid; Orobanchaceae; parasitic plants; Stockwell's bleach.

A haustorium is the unique organ of parasitism found in parasitic angiosperms, providing a physical and physiological link from parasite to host plant. One of the difficulties of research on haustoria in Orobanchaceae is that preserved tissues are often darkly stained, which requires clearing prior to experimentation. While isolating haustoria for plastic and paraffin sectioning (preserving in glutaraldehyde), a duplicate set of haustoria were also preserved in 70% ethanol for whole mount staining with aniline blue to detect callose using fluorescence microscopy. Initial attempts to detect callose were thwarted by the presence of obscuring dark compounds in the fixed tissues, presumed to be tannins. Most clearing techniques (Sporne, 1948; Lersten, 1967; Morley, 1968; Herr, 1971; Ruzin, 1999) have been developed for leaf, seed, embryo, and young seedling materials that have less or different secondary products that obscure tissues. Routine clearing protocols were not successful in lessening or removing tannins from intact haustoria, which are delicate structures. In addition to those tannins commonly encountered in plant tissues, Orobanchaceae contain iridoid glucosides (Rank et al., 2004), compounds thought to be responsible for turning specimens of Orobanchaceae black upon drying. Iridoids could also be involved in darkening of preserved haustoria.

To develop a method to effectively clear haustoria, seven samples (representing four species) of ethanol-fixed haustoria were subjected to various treatments as described below. First, clearings were attempted with Herr's 4½ solution (Herr, 1971) for up to two weeks at room temperature without success. Second, the same haustoria were then placed in a 1% solution of KOH at room temperature for 24 h; there was no visible coloration change during this time. Next, the haustoria were transferred to a room temperature solution of lactic acid saturated with chloral hydrate for 5 d. After no visual improvement, vials were transferred to a 42°C oven for 4 d. Again, there was no decrease in coloration. Finally, the haustoria were transferred from the 42°C lactic acid–chloral hydrate solution back to Herr's 4½ solution, and left at room temperature for two months, again with no change. As a final attempt, a separate collection of haustoria were placed in 5% NaOH at 50°C for two weeks. While there was some reduction in coloration after the full two-week treatment, there was significant tissue degradation (maceration), rendering the haustoria unsuitable for subsequent experiments (further clearing would have been necessary had the tissues remained intact). At this point it became clear that another strategy was needed, as these clearing methods had failed to reduce tissue coloration, or had degraded tissue integrity.

The use of Stockwell's bleach to remove tannins from plant tissues was explored by Schmid (1977) utilizing a diversity of plant tissue. A precursor of Stockwell's bleach was first used by Stockwell (1934) to pretreat slides with a 1% aqueous chromic acid solution prior to staining. The complete recipe and protocol was first published by Johansen (1940), and the bleaching is described as follows: "the chromic acid renders the precipitated tannins soluble, the acetic acid then removes them, and the dichromate thereupon catalyzes the tissues." Among the advantages of this procedure, Schmid (1977) lists: the bleach can be effectively used with a wide variety of materials including herbarium material and on tissues preserved using a variety of fixatives, it effectively removes most or all of the obscuring tannins,

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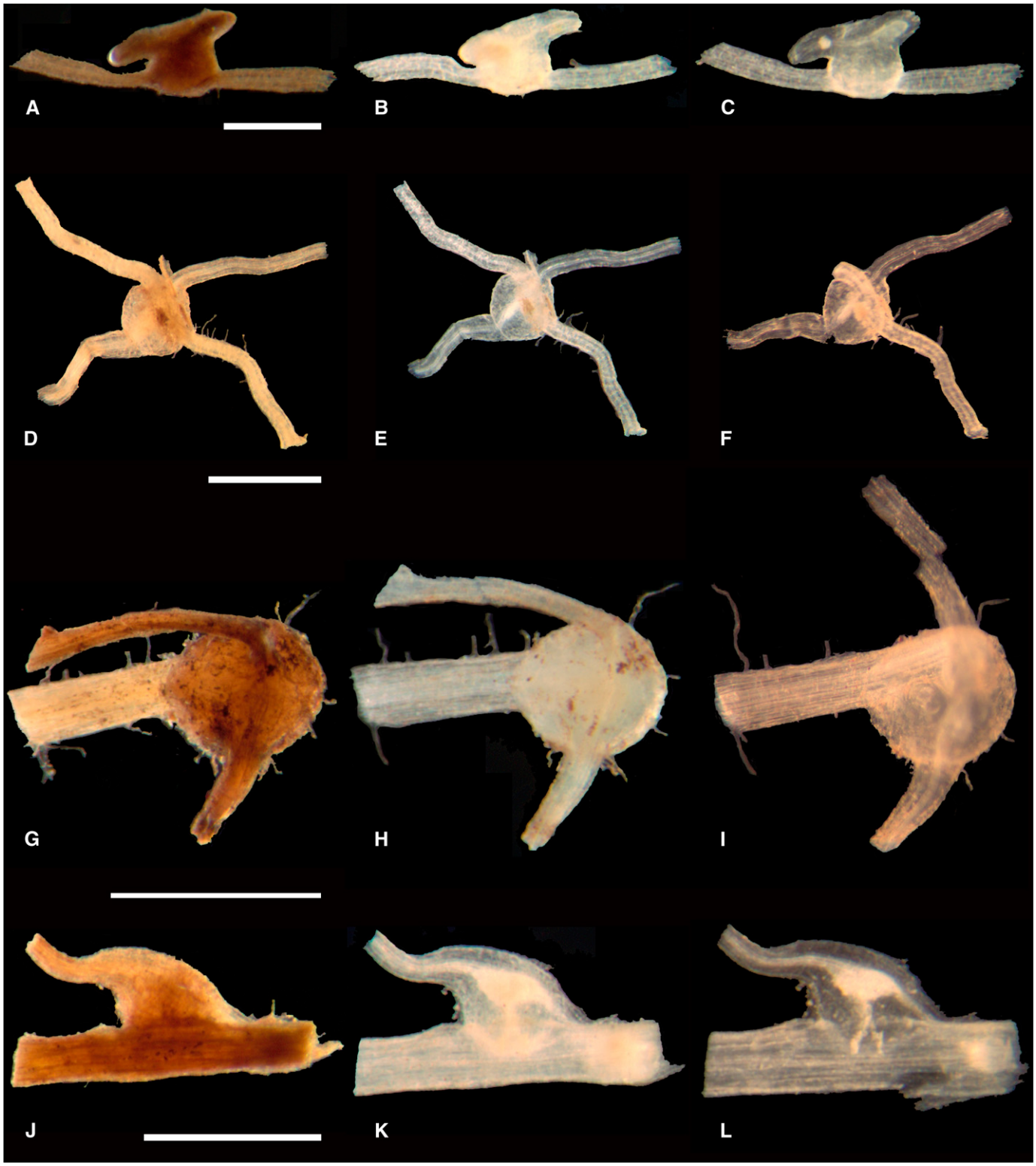

Fig. 1. The effects of Stockwell's bleach and lactic acid–chloral hydrate on haustoria: (A–C) *Bellardia trixago*, (D–F) *Castilleja exserta*, (G–I) *Castilleja foliolosa*, and (J–L) *Parentucellia latifolia*. A, D, G, J: haustoria fixed in 70% EtOH prior to treatment. B, E, H, K: haustoria after treatment in Stockwell's bleach (3, 4, 5, and 3 d, respectively). C, F, I, L: haustoria after further treatment in lactic acid saturated with chloral hydrate at 42°C. Scale bars = 1 mm.

it uniformly affects tissues such that subsequent staining times are unaffected, and it enhances stained materials through increased contrast of tissues. Schmid used Stockwell's bleach on sectioned material already mounted on slides and suggested that "none of these advantages apply to the bleaching of whole objects" (Schmid, 1977). His results on sectioned tissues were intriguing, which motivated the current study using Stockwell's bleach on whole haustoria prior to clearing.

METHODS AND RESULTS

Stockwell's bleach was mixed following the proportions used by previous investigators (Johansen, 1940; Foster and Gifford, 1947; Schmid, 1977; Ruzin, 1999), but substituting a 10% chromic acid solution for the cited 1 g of powdered chromic acid, yielding the same final 1% concentration:

10 mL 10% chromic acid (LabChem, cat. no. LC131001)
10 mL glacial acetic acid (Fisher Scientific, cat. no. BP2401-212)
1 g potassium dichromate (Sigma-Aldrich, cat. no. 60188)
80 mL dH_2O

This protocol was tested in the following four taxa: *Bellardia trixago* (L.) All., *Castilleja exserta* (A. Heller) T. I. Chuang & Heckard, *C. foliolosa* Hook. & Arn., and *Parentucellia latifolia* (L.) Caruel (Appendix 1). These accessions were selected primarily because of the high intensity of darkly staining compounds present in preserved haustoria (compared to other accessions with less intensely darkly stained haustoria), which would provide a robust test of the protocol's efficacy. Further, the two species of *Castilleja* were chosen to represent two different habits (annual vs. perennial). Three complete sets of these haustoria (of all four species) were successively subjected to this protocol. All tissues used in this study were fixed in 70% EtOH.

Haustoria were bleached for up to 10 d until all coloration was removed, leaving the tissue opaque white to (rarely) translucent. Most haustoria were completely bleached in 3–5 d. The bleach solution was changed daily if it showed discoloration (turning from orange to brown), which was typical for the first 3–4 d. After that time, the bleach typically became discolored after ~2 d, at which point it was replaced. Once tissues became opaque white or translucent, they were washed three times with dH_2O, then dehydrated in a graded ethanol series (minimum 2 h each: 50%, 70%, 95%, 3× 100%), and placed in a solution of lactic acid saturated with chloral hydrate at 42°C until cleared (3 d). After successful clearing, the tissues were washed three times in 100% EtOH, rehydrated, and stored in 70% EtOH. Haustoria were photographed using an Olympus SZH stereomicroscope (Olympus, Center Valley, Pennsylvania, USA) fitted with a SPOT RT Color CCD camera (model 2.2.1) (Diagnostic Instruments, Sterling Heights, Michigan, USA) using transmitted light.

In all cases, haustoria were translucent after the subsequent step of clearing in lactic acid saturated with chloral hydrate (Fig. 1C, F, I, L). There was dramatic improvement in tannin reduction in all haustoria treated (Fig. 1). After bleaching, haustoria were white (Fig. 1B, H) or translucent (Fig. 1E, K). The translucency of all haustoria, including those appearing translucent after bleaching, was further improved with the lactic acid–chloral hydrate treatment (Fig. 1F, L).

Subsequent to the trials presented here, haustoria representing a wider taxonomic sampling from Orobanchaceae were successfully cleared with this two-step protocol (results not shown). Further, initial experiments with aniline blue staining on cleared haustoria positively detected callose, indicating that the bleaching and clearing did not negatively affect the ability of callose to bind aniline blue (results not shown).

Appendix 1. Specimens used in this study. Information presented: taxon; voucher specimen, collection locale; herbarium.

Bellardia trixago; *Randle 217*, TX, USA; SHST. *Castilleja exserta*; *Morawetz 495*, CA, USA; RSA. *Castilleja foliolosa*; *Morawetz 497*, CA, USA; RSA. *Parentucellia latifolia*; *Morawetz 502*, CA, USA; RSA.

CONCLUSIONS

Stockwell's bleach effectively removes tannins from haustoria in Orobanchaceae. When Stockwell's bleach is employed prior to a classical clearing method, tissues with tannins can be successfully cleared (Fig. 1). With the set of haustoria documented for this paper, two specimens appeared translucent after bleaching (Fig. 1E, K). Despite the vast improvement over their unbleached condition, these specimens still benefitted from subsequent clearing with the lactic acid–chloral hydrate treatment, revealing more detail of the haustorial vascular system (Fig. 1F, L). Notably, after bleaching and clearing, one can observe the well-developed vascular core of the haustorium (Fig. 1L) and xylem bridges between parasite and host vascular systems (Fig. 1L). One disadvantage of this method is that haustoria treated with Stockwell's bleach become brittle (note broken host root in Fig. 1F compared to Fig. 1E). However, this consequence of increased brittleness is outweighed by the benefit of obtaining cleared tissues for subsequent investigations, such as callose staining and three-dimensional reconstruction using confocal microscopy. The success of this two-step clearing protocol is promising for other recalcitrant tissues that are unable to be cleared with classical methods alone.

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